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PRINCIPAL INVESTIGATOR: Philip S. Zeitler, M.D., Ph.D.

CONTRACTING ORGANIZATION: University of Colorado Health Sciences Center
Denver, Colorado 80045-0508

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Philip S. Zeitler, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Colorado Health Sciences Center
Denver, Colorado 80045-0508

E-Mail: Phil.Zeitler@uchsc.edu

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13. ABSTRACT (Maximum 200 Words)

. We hypothesize that ***GHRH functions as an autocrine/paracrine growth factor in neoplastic breast tissue***. To address this hypothesis, we have undertaken a comprehensive examination of the physiology of GHRH in immortalized breast cancer cell lines. We report here the results of the project. The data summarized here indicate that endogenous GHRH acts as a growth factor through activation of MAPK/ERK (in a ras and raf dependent fashion). In addition, the data suggest an anti-apoptotic action of GHRH through suppression of p38 activation of a caspase cascade and consequent inhibition of Bcl-2 cleavage. Activation of an independent Jnk pathway may antagonize the effects of GHRH on the p38 pathway. The data also indicate a possible reciprocal autocrine/paracrine role for GHRH and somatostatin in the regulation of breast tumor cell growth. Finally, the data support a role for tightly regulated GHRH secretion as an important component in the promotion of breast tumor cell growth. The emerging picture of the pathway by which GHRH promotes growth and inhibits apoptosis in breast cancer cell lines furthers our understanding of the previously demonstrated actions of GHRH antagonists to inhibit breast cancer growth *in vitro* and *in vivo*. More importantly, this understanding begins to suggest ways in which GHRH antagonists might fit into therapeutic regimens, as pro-apoptotic agents in their own right or as adjuvant agents supporting the action of traditional anti-neoplastics.

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INTRODUCTION

Growth hormone-releasing hormone (GHRH), in addition to stimulating the release of growth hormone (GH) from the pituitary, acts as a trophic factor for pituitary somatotrophs[1-8]. In addition, GHRH is expressed outside of the hypothalamic/pituitary axis, with significant expression in the gonads, gastrointestinal tract, pancreas, thymus, and lymphocytes[9-14]. Expression has also been reported in a variety of tumors[15], including pancreas, lung, CNS[16] and, recently, breast, prostate, and endometrium[17-19]. While the role of extrahypothalamic GHRH is unknown, mitogenic effects have been demonstrated on lymphocytes and germ cells *in vitro*[20,21]. The current study examines the hypothesis that ***GHRH functions as an autocrine/paracrine growth factor in neoplastic breast tissue.*** To address this hypothesis, we have undertaken a comprehensive examination of the physiology of GHRH in immortalized breast cancer cell lines. We examine the effect on cell growth and proliferation of exogenous GHRH and disruption of endogenous GHRH with specific inhibitors. We also identify the intracellular signaling pathways that mediate the effects of GHRH on breast cancer cells. Finally, we will dissect the mechanism by which GHRH and its receptor begin to be expressed in the process of neoplastic transformation of breast tissue. We report here the results of the course of this project. During the first 12 months of the project, we demonstrated that disruption of endogenous GHRH inhibits proliferation and stimulates apoptosis in breast cancer cell lines and that this effect is mediated through a pathway involving the MAP kinases, ERK ½, p38 and JunK, as well as the caspase cascade. During the second 12 months of the project, we expanded the investigations, with particular attention to the participation of the p38 pathway, intracellular caspases, and the pro-survival protein Bcl-2. We have also demonstrated that MAP kinase, through a raf/ras dependent pathway, mediates the growth promoting effects of GHRH in breast cancer cell lines. In the final 12 months of the project, we have further expanded this work to demonstrate that both exogenous and endogenous GHRH stimulates cell proliferation and inhibits apoptosis in MDA231 cells, that endogenous GHRH secretion is coupled to a cycle of endogenous cellular activation suggesting the activity of important autocrine/paracrine control of cell growth by GHRH, and that somatostatin may play a modulatory role in this cycle.

BODY

Task 1: To determine the relationship between expression of GHRH, expression of GHRH receptor, and cellular proliferation in breast cancer cell lines (Months 1 - 15)

Quantitate the expression of protein and mRNA for GHRH and GHRH receptor in breast cancer cell lines (Months 1 - 4)

We have continued to be stymied by the technical problems previously described, namely the unavailability of reagents for the approaches originally proposed. In the absence of information regarding expression of these genes in other breast cancer cell lines, work has continued to focus on the physiology of MDA231 cells, which express both GHRH and the GHRH receptor at high levels.

Define the effect of GHRH and its antagonists on the growth, proliferation, and apoptosis of breast cancer cells (Months 4 -10)

Task completed – Manuscript published (Zeitler P, Siriwardana G. Antagonism of endogenous growth hormone-releasing hormone leads to reduced proliferation and increased apoptosis in MDA231 breast cancer cells. Endocrine 18:85-90, 2002) and included in Appendix.

The aim of this task was to determine the physiologic effect of disruption of normal GHRH secretion and action on the proliferation and survival of breast cancer cell lines. Because of the high level of expression of both GHRH and its receptor in MDA231 cells relative to other cell types so far evaluated, these experiments focus predominately on this cell line.

MDA231 cells, originally obtained from ATCC, were grown to confluency under standard conditions in DMEM supplemented with 10% FCS. Prior to experiments, the cells were lifted with PBS/2%EDTA, plated at 8,000/cm in 96-well, 24-well, or 6-well plates, and allowed to attach overnight in DMEM/2%FCS. The competitive GHRH antagonist [N-acetyl-Tyr1, D-Arg2] fragment 1-29Amide(Sigma)(GHRHa) was dissolved in 2%acetic acid/1%insulin-free BSA to a stock concentration of 1 mM. GHRHa was added in 80μl DMEM and allowed to incubate for 1 hour. Control cells were treated with the same final concentration of vehicle alone. The medium was then brought to 2%FCS and allowed to incubate until the indicated times. At the time of counting, the medium was aspirated, the cells lifted with 50μl trypsin, and resuspended in 150 μl of PBS. Four 0.1μl samples of each well were counted by hemocytometer, with 8 replicates per treatment.

As shown in figure 1, a single treatment of MDA231 cells with 3 μM antagonist resulted in an approximately 25% decrease in cell number after 24 hours. Subsequently, cell numbers increased in parallel with control cells, indicating that the effect is transient and reversible. A second treatment after 24 hours led to an additional 24 hours of inhibition of the increase in cell number (not shown).

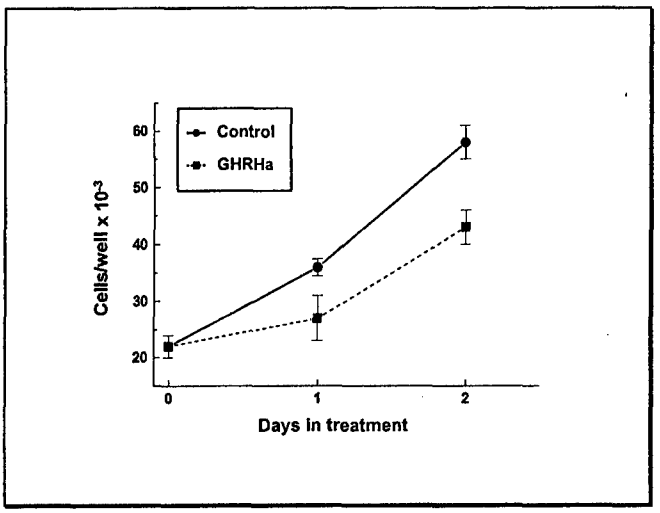

Figure 1: Effect of 3μM GHRHa on MDA231 cell counts *in vitro.* Values represent the mean ± SEM, n = 8 replicates at each time point for each treatment

The inhibition of cell number increase by GHRHa is also dose dependent. As shown in figure 2, exposure for 24 hours to GHRHa resulted in decreases in cell number ranging from 6% at 40 nM to 25% at 5 μM. Higher doses did not lead to decreases greater than 25%.

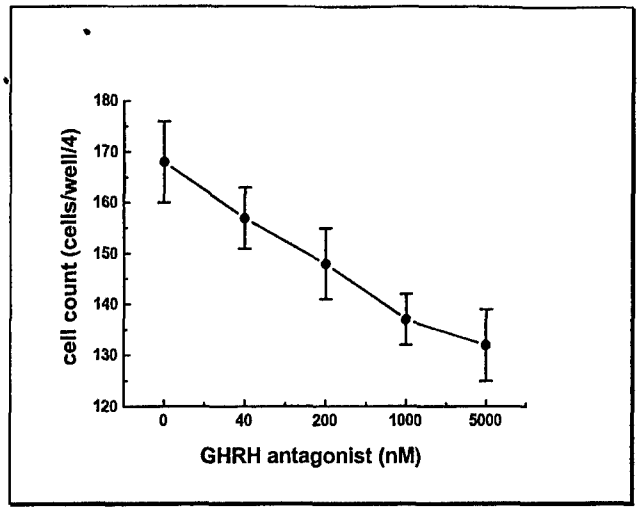

Figure 2: Dose dependent effect of GHRHa on MDA231 cell counts in vitro. Values represent the mean ± SEM, n = 8 replicates at each time point for each treatment.

In order to determine whether the effect of disruption of endogenous GHRH on cell number resulted from decreased cell proliferation, we examined the effect of GHRH antagonism on uptake of tritiated thymidine. MDA231 cells were grown in 100µl DMEM/2%FCS in a 96 well plate overnight. GHRHa was added in 80µl DMEM and allowed to incubate for 1 hour. The medium was then brought to 2%FCS. Four hours after GHRHa treatment, 0.8µl ^{3}H-thymidine was added to each well. At the indicated times, cells were washed thrice with 150µl PBS, followed by 25µl 10%TCA. After 5 minutes, 100µl 0.1M NaOH was added followed by 27.5 µl 0.1M HCl. The entire content of the well was transferred to scintillation counter tubes and counted for 10 minutes. Each treatment was examined in 8 replicates.

As shown in figure 3, exposure of MDA231 cells to a single dose of 3 µM GHRHa resulted in a rapid and transient decline in thymidine uptake followed by uptake parallel to control cells. This change in thymidine uptake indicates a decrease in DNA synthesis and suggests a decrease in cellular proliferation. When cells were exposed to a second dose of GHRHa after 4 hours, tritiated thymidine uptake was inhibited for an additional 4 hours followed by recovery and uptake parallel to control cells.

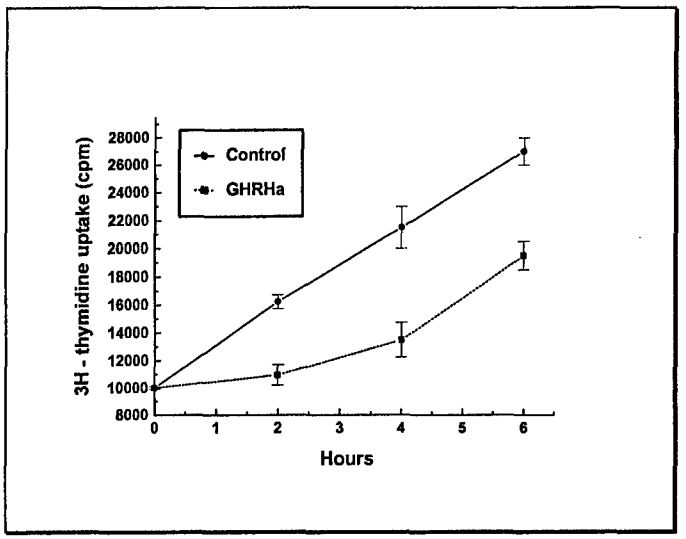

Figure 3: Effect of 3µM GHRHa on ^{3}H-thymidine uptake by MDA231 cells *in vitro* Values represent the mean ± SEM, n = 8 replicates at each time point for each treatment.

To determine whether antagonism of endogenous GHRH also decreased cell counts through promotion of cellular apoptosis we examined the effect of GHRHa on apoptosis using two independent techniques. First, we examined the effect of GHRHa on DNA laddering. Cells were grown in 3.5cm plates overnight. Following treatment with GHRHa, the volume of medium was

increased to 3 ml. Cells were harvested 12 hours after GHRHa treatment, pelleted by centrifugation, and washed once in PBS. Cells were lysed in 20μl lysis buffer (50 mM Tris pH 8.0, 10 mM EDTA, 0.5% SDS, 0.5 mg/ml proteinase K) and heated to 50C for 1 hour. The mixture was then heated to 90C for 3 min to deactivate the proteinase K, treated with 10 μl RNAse A to a final concentration of 0.5 μg/ml in TE and heated to 50C for 1 hour. Samples were separated by electrophoresis through 2% agarose with ethidium bromide and visualized (Alphaimager).

As shown in figure 4, exposure of MDA231 cells to 3 μM GHRHa lead to a marked increase in DNA laddering compared to vehicle alone, indicating increased apoptosis.

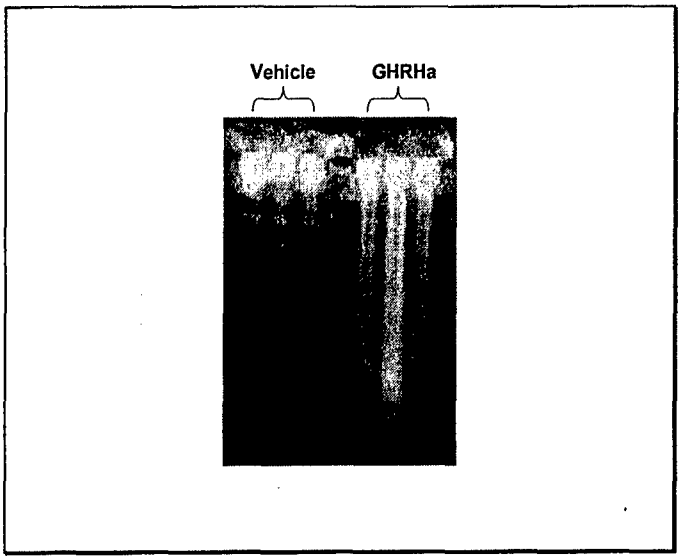

Figure 4: Effect of 3 μM GHRHa on DNA laddering in MDA231 cells. Each lane represents the total DNA sample obtained from a single replicate, 3 replicates per treatment.

Next, we examined the effect of GHRHa on nuclear condensation, another measure of apoptosis. Cells were grown in 100μl DMEM/2%FCS on chamber slides overnight. GHRHa was added in 80μl DMEM and allowed to incubate for 1 hour. The medium was then brought to 2%FCS. Twenty-four hours after GHRHa treatment, cells were washed briefly with PBS, fixed in 4% paraformaldehyde for 10 min, followed by 70% EtOH in glycine buffer for 10 min at -20C. Cells were then washed in PBS, incubated with Hoechst dye (8μg/ml) for 15 min at RT, and rinsed three times in PBS. Slides were then blinded and cells were visualized by fluorescent microscopy and apoptotic cells counted (4 fields per slide, eight slides per treatment). (100 X magnification). Exposure of MDA231 cells to 3 μM GHRHa for 24 hours increased the frequency of appearance of condensed nuclei after staining with Hoechst dye. To quantify this increase, we counted the number of condensed nuclei present in a 100X field (4 fields per slide, 8 slides per treatment). As shown in two experiments in figure 5, MDA231 cells exposed to vehicle alone had approximately 30 apoptotic cells per field. However, after 24 hours in the presence of 3 μM GHRHa, the frequency of apoptotic cells increased by 60%.

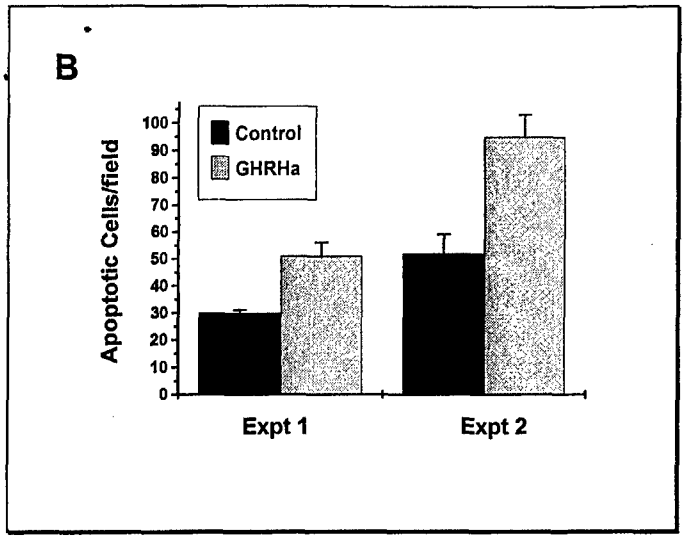

Figure 5: Effect of 3 μM GHRHa on nuclear condensation of MDA231 cells *in vitro.* Each value represents the mean ± SD, n = 8 replicates per treatment, 4 fields counted per replicate.

Exposure of MDA231 cells to GHRHa for 24 hours leads to an approximately 60% increase in the incidence of apoptotic MDA231 cells. However, the total incidence of apoptosis appears to be relatively small, consistent with the previously demonstrated 25% decrease in cell number at 24 hours.

Finally, cells were plated as described, and exposed to GHRH antagonist (3 μM) or vehicle for 24 hours. Cells were then harvested, stained with Krishan's stain, and separated by fluorescent cell sorting. As shown in figure 7, exposure of MDA231 cells to GHRH antagonist results in a marked increase in cells demonstrating apoptotic nuclear signals.

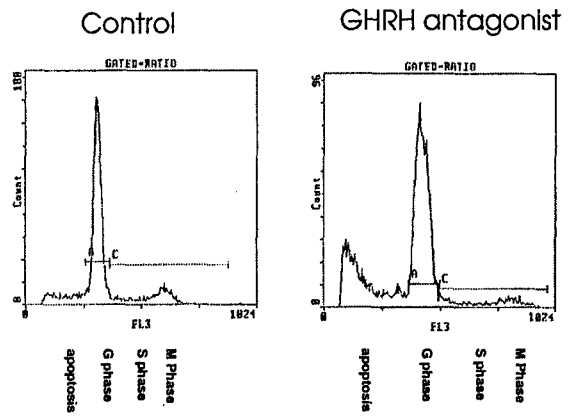

Figure 6: Effect of 3 μM GHRH antagonist on apoptosis determined by FACS analysis in MDA231 cells.

In order to confirm the specificity of these effects of GHRHa, we also examined the effect of additional peptide GHRH antagonists of entirely different structure obtained from Dr. David Coy at Tulane University, peptides PRL 2194 and PRL2640. These peptides have greater affinity for the GHRH receptor than GHRHa. Lyophilized peptides were dissolved in water and added to media at the concentrations shown using an experimental paradigm identical to that used for GHRHa. As seen in figure 8, both of these antagonists lead to a marked dose-dependent decrease in cell number 24 hours after treatment. Furthermore, these peptides with greater affinity for the GHRH receptor than GHRHa have an efficacy that is also greater than GHRHa, leading to cell reductions of up to 65%, compared to 15-25%. The observation that these alternative antagonists, with a structure different from GHRHa reduce cell proliferation and that the degree of reduction correlates crudely with binding affinity further support the conclusion that disruption of endogenous GHRH action impairs normal breast cell proliferation and this effect is mediated through the GHRH receptor itself.

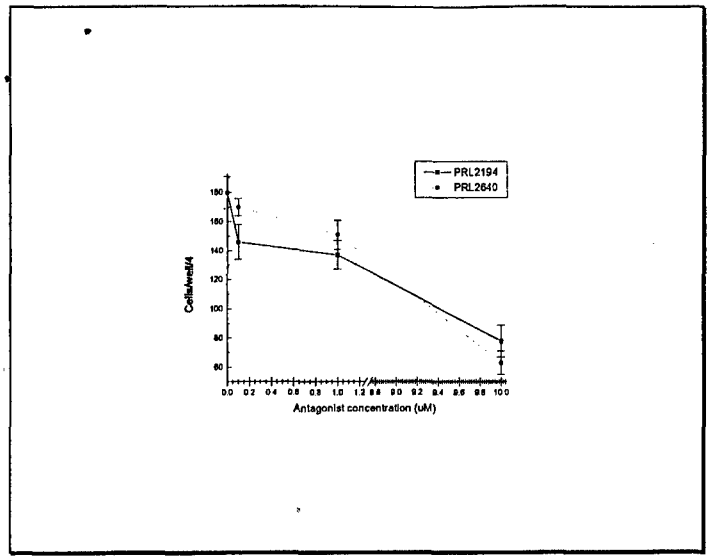

Figure 7: The does dependent effect of peptide GHRH antagonists, PRL2194 and PRL 2 640 o n M DA231 cell c ounts *in v itro.* Each value represents the mean ± SD, n = 8 replicates per treatment.

Taken together, the data produced as part of this project during the last 12 months indicate that antagonism of the endogenous GHRH autocrine/paracrine system in MDA231 breast cancer cells leads to inhibition of cell proliferation, as well as increased cellular apoptosis, the combination of which leads to decreased cell number. The effect of GHRH antagonists are dose-dependent, transient, and reversible. In addition, the data imply that the effect of GHRH antagonists are mediated through GHRHr itself, rather than cross reaction at related receptors. These results are consistent with previous demonstrations of an inhibitory effect of GHRH antagonists on a variety of reproductive and GI tract tumors[22-26] and extend these observations to provide information regarding the mechanism of the effect of GHRH antagonists. The results also confirm that breast cell lines *in vitro* provide a useful model system in which to further investigate the physiology of GHRH in breast tumors.

Determine whether changing the expression of GHRH and/or GHRH receptor in breast cancer cell lines results in changes in cell growth characteristics (Months 10-15).

The proposed transfections have been attempted but have not been satisfactorily successful for technical reasons. No evaluable data are available at the time of this report.

Task 2: To determine the intracellular signaling pathway(s) that mediate the effects of GHRH on growth in breast cancer cells (Months 15 - 24)

Define t he g eneration o f c AMP a nd a ctivated M AP k inase p athway i ntermediates i n b reast cancer cells in response to GHRH (Months 15-18)
Determine t he p athway(s) t hat m ediates t he p roliferative a nd/or a poptotic e ffects o f G HRH (Months 19-24)

The work proposed in this task has continued to progress significantly over the last year, with continued expansion of our understanding of the pathways involved in transduction of the GHRH signal in MDA231 cells. One manuscript has been submitted for review and another is in preparation.

We had previously demonstrated that GHRH, in addition to stimulating the intracellular synthesis of cAMP, also activates the MAPK (ERK ½) pathway in pituitary cells[27]. Until this demonstration, it had been a ssumed t hat t he p roliferative a ctions of G HRH o n p ituitary s omatotroph (GH-secreting c ell) were mediated by cAMP generation, as are the hormone releasing actions. However, cAMP

stimulation of proliferation is uncommon, since cAMP is much more commonly associated with suppression of cell proliferation. Therefore, the identification of involvement of the MAP kinase pathway in GHRH action, a pathway well known to be associated with cellular proliferation, has helped to clarify the mechanism of GHRH effects on cell proliferation.

Therefore, we examined the effect of GHRH on components of the MAPK pathway. Initial experiments focused on the effects of exogenous GHRH on MDA231 cells. However, it became quickly clear that basal levels of ERK 1/2 activation were significant, likely reflecting the actions of endogenous GHRH. Attempts to decrease basal MAPK activation by manipulation of culture conditions were initially unsuccessful. While it was possible to demonstrate a modest decrease in ERK 1/2 activation in the presence of GHRHa, the results were less than compelling. We returned to this problem in year 3 and the results are discussed more fully below.

Since we had demonstrated that antagonism of GHRH leads to increased apoptosis, we turned our attention to other components of the MAPK pathway known to be related to this phenomenon[28-30]. Since activation of these components increases under conditions that promote apoptosis, we thought it likely that demonstration of these positive changes would be more straightforward than documenting the negative changes of ERK1/2. This proved to be the case.

MDA231 cells were exposed to GHRH antagonist (3 μM) or vehicle for 20 min. Cells were then washed, lysed, and the proteins separated by PAGE and analyzed by Western blot hybridization with phospho-specific Jun kinase or Phospho-specific p38 kinase antibody. As shown in figure 8, exposure to GHRH antagonist resulted in marked activation/phosphorylation of both JunK and p38 kinase compared to vehicle.

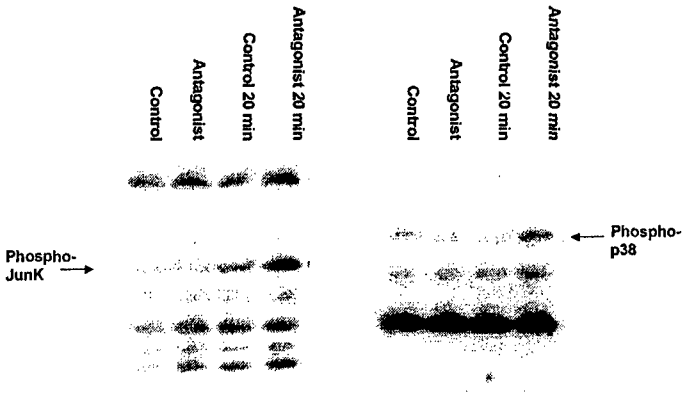

Figure 8: Effect of 3 μM GHRH antagonist on phosphorylation of Jun kinase (left panel) and p38 kinase (right panel) in MDA231 cells.

Activation of the p38 pathway has been associated with initiation of apoptosis in a number of cell systems, while activation of JunK has a more complicated relationship to apoptosis, associated with both stimulation and suppression of apoptosis[28-30].

We next examined the time course of P38 activation in response to GHRHa, using the same experimental paradigm. As shown in figure 9, exposure of MDA231 cells to 3 μM GHRHa leads to dramatic, rapid, and transient phosphorylation/activation of P38, with onset within 20 minutes and return to baseline before 2 hours. This pattern of P38 activation has been seen in other apoptotic systems.

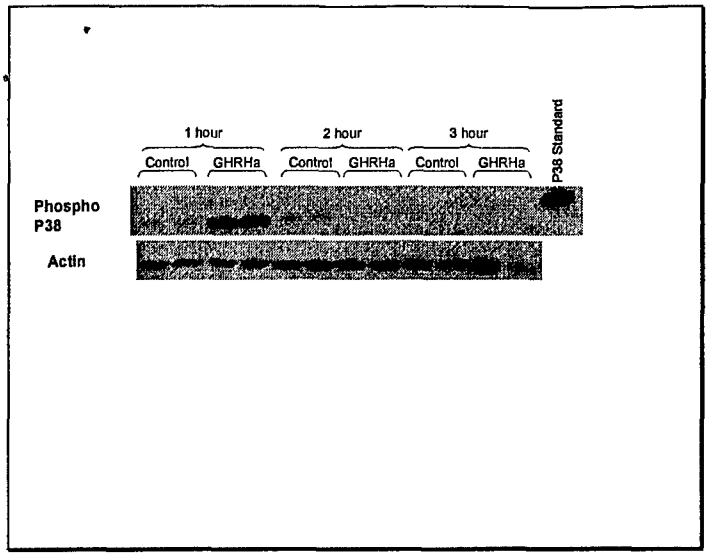

Figure 9: Time course of the effect of 3 μM GHRH antagonist on phosphorylation of p38 kinase in MDA231 cells

To confirm independently the effect of GHRH antagonism on p38 phosphorylation, we examined the effect of a potent, non-competitive GHRH antagonist, PRL 2140 (David Coy, Tulane University) using the same paradigm. As seen in Figure 10, this independent antagonist shows essentially the same effect on p38 phosphorylation as GHRHa.

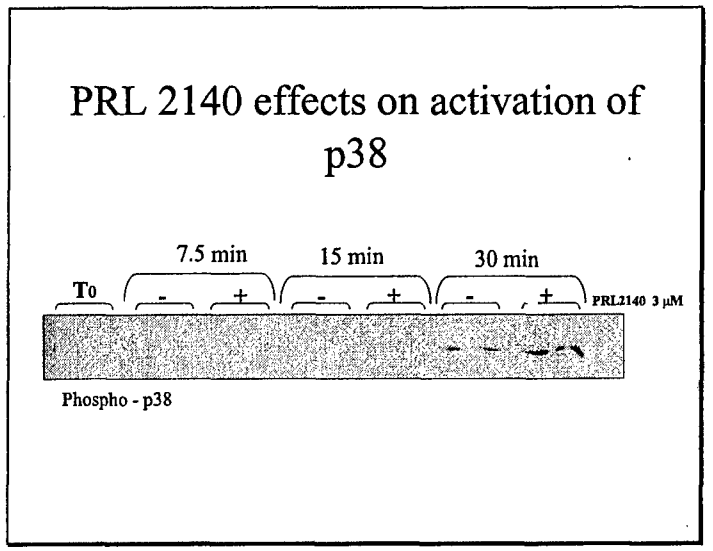

Figure 10: Time course of the effect of the GHRH antagonist PRL 2140 on phosphorylation of p38 kinase in MDA231 cells

In order to determine whether activation of P38 by GHRHa is related to apopotosis, we examined the effect of the P38 inhibitor SB203580 on cell number reduction and apopotosis in response to treatment with GHRHa. MDA231 cells were pretreated with 10 μM SB203580 30 minutes prior to exposure to GHRHa and cell counting, as described above. As shown in figure 11, inhibition of P38 activation by the SB compound prevented the decrease in cell number seen in response to GHRHa, strongly suggesting that activation of P38 mediates the effect of GHRHa on changes in cell number.

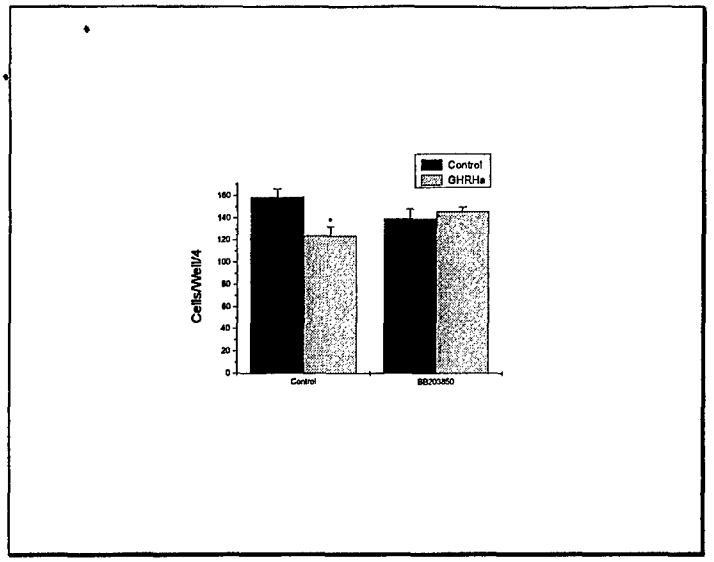

Figure 11: Effect of P38 inhibition on the GHRHa induced decrease in MDA231 cell number. *Each value represents the mean ± SD, n = 8 replicates per treatment. * = P<0.05*

We next examined the effect of P38 inhibition on DNA laddering in response to GHRHa treatment of MDA231 cells. Again, cells were pretreated with SB203580 30 minutes prior to exposure to GHRHa and cells processed for DNA laddering as described above. As seen in Figure 12, inhibition of P38 activation by the SB compound prevented the stimulation of DNA laddering seen in response to GHRHa, suggesting that P38 activation is required for the GHRHa stimulation of apopotosis.

Figure 12: Effect of P38 inhibition on the GHRHa induced apoptosis in MDA231 cells. *Each lane represents the total DNA sample obtained from a single replicate, 2 replicates per treatment.*

As noted above, Jun Kinase has a more complicated relationship to apoptosis, being associated with both stimulatory and inhibitory actions under different circumstances. We next examined the effect of inhibition of Jun Kinase on the response of MDA231 cell numbers to GHRHa. MDA231 cells were transfected with 5 μg of a vector containing a CMV promoter-driven dominant-negative Jun Kinase 1 (Jnk1 APF) or Jun Kinase 2 (Jnk2 APF) construct by electroporation, grown for 24 hours in serum containing medium and then treated with GHRHa as described above. As seen in figure 13, exposure of MDA231 cells to GHRHa in this experiment resulted in a modest (14%) decrease in cell number after 24 hours. Transfection with dominant negative JnK 1 had no effect on this response to GHRHa. However, transfection with dominant negative JnK 2 more than doubled the decrease (39%). Pretreatment of cells with SB203580 completely prevented the decrease in cell number following exposure to GHRHa, even in cells transfected with dominant-negative Jnk 2 (not shown). The amplification of the effect of GHRHa when JnK 2 is inhibited suggests that activation of JnK 2 antagonizes the effect of P38 activation on cell number.

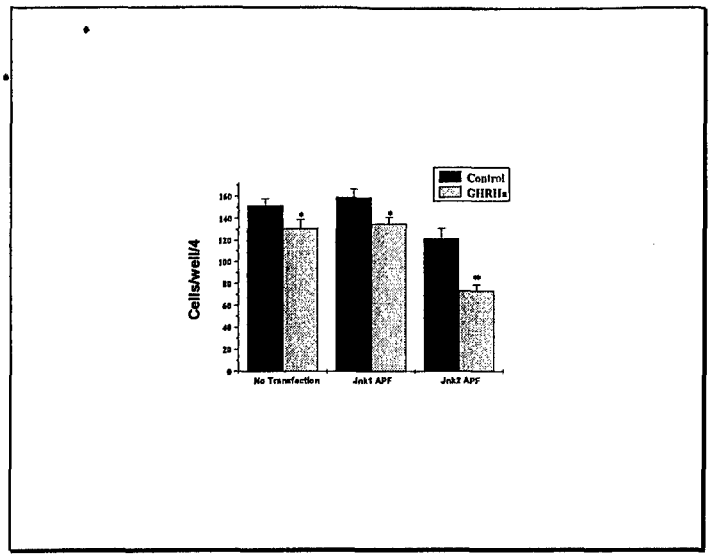

Figure 13: Effect of Jun Kinase 1 and Jun Kinase 2 inhibition on the GHRHa induced decrease in MDA231 cell number. Each value represents the mean ± SD, n = 8 replicates per treatment. * = P<0.05, ** = P<0.01

This experiment suggests that disruption of endogenous GHRH action on MDA231 cells activates both P38 and JunK 2, the former stimulating and the latter inhibiting apoptosis. It is intriguing to hypothesize that the physiologic state of the cell at the time of GHRH disruption influences the relative degree of activation of these two pathways, leading to alterations in the eventual response of cell proliferation and apoptosis.

Taken together, the experiments described up to this point indicate that disruption of endogenous GHRH action on MDA231 cells leads to activation of P38 and Jnk2, along with inhibition of ERK ½. In addition, the effects of GHRH disruption on both cell number and apoptosis are mediated through activation of P38, with Jnk2 perhaps playing a modulatory role. Thus, we can conclude that endogenous GHRH functions in MDA231 cells to stimulate ERK ½ and inhibit activation of P38, thereby promoting cell proliferation and limiting apoptosis.

We next examined downstream components of the p38 pathway leading to apoptosis in response to blockade of endogenous GHRH. First, in order to determine the participation of caspases in this process, MDA231 cells were treated with GHRHa (5 uM) as in previous experiments. The lysate was subjected to SDS PAGE Electrophoresis, and transfer to Immobilon-P membrane. After blocking, the 12 kDa caspase-3 small subunit was detected using a caspse-3 specific antibody. As shown in figure 14, the phosphorylation of p38 approximately 1 hour after exposure to GHRHa, seen in previous experiments, is accompanied by a marked increase in activated caspase 3 after approximately 1 hour, followed by a reduction to basal levels.

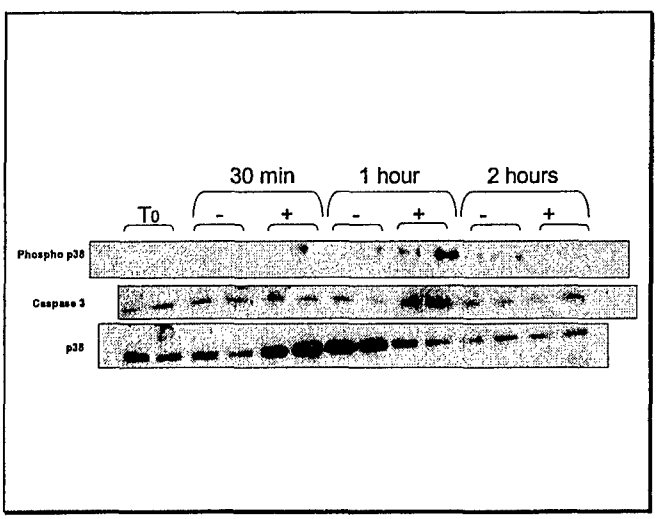

Figure 14: Antagonism of endogenous GHRH with GHRHa (5 uM) promotes p38 phosphorylation and activation of caspase 3.

In order to confirm the participation of caspase family members, we examined the effect of caspase inhibition on the cell number reduction following antagonism of endogenous GHRH. MDA231 cells were pretreated with caspase inhibitors 30 minutes prior to exposure to GHRHa, followed by cell counting as described above. In this experiment (Figure 15), antagonism of caspases 2, 3, and 9, but not 1,4,6,and 8 prevented the reduction in cell numbers seen 24 hours after exposure to GHRH antagonist, suggesting that activation of caspases, particularly some combination of 2,3 and 9 is required for the effect on apoptosis.

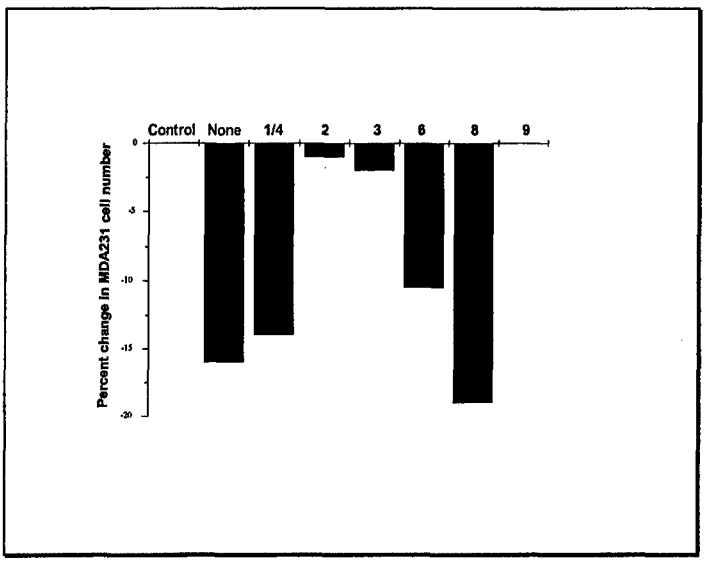

Figure 15: Inhibition of Caspase 2,3 and 9 prevents the GHRHa induced decrease in MDA231 cell number. (n = 4 per group).

Although we have not yet determined the order in which the caspases -2, -3 and -9 are activated, the available literature suggests that caspase-9 is likely to be the initiator caspase in the GHRH antagonized MDA231 cells[31]. In other words, these data suggest that endogenous GHRH in MDA 231 cells blocks a caspase-9 based apoptotic cascade that otherwise would occur. It has been shown that caspase-2 is a direct effector of mitochondrial apoptosis and is inactive towards other caspase zymogens[32]. Similarly, in several human cell lines, apoptosis occurred through an early phase of mitochondrial dysfunction via caspase-2. However, activation of caspase-3 was necessary in these cells for the activation of caspase-2[33]. Thus, since Caspases -2 and -3 are known to be effector caspases, it is reasonable to assume that caspase -2 and -3 act as effectors in our system. *In vitro*, caspase-2 is the preferred cleavage substrate for caspase-3. Thus, it appears that caspase-3 is responsible for the activation of caspase-2 and not *vice versa*[31,33]. Taken together, the data suggest that antagonism of endogenous GHRH leads to activation of a casade from caspase 9 to 3 to 2.

The ratio of pro survival Bcl-2 to pro apoptotic Bcl-2 provides a major regulator of apoptosis[34]. In order to examine the effect of GHRH antagonism on BCL-2 activity, MDA231 cells were treated with GHRHa (5 uM) as in previous experiments. The lysate was subjected to SDS PAGE and western analysis for the 29 KDa pro-survival BCl-2 protein using Bcl-2-specific antibody. As seen in Figure 16, caspase 3 activation occurs, as previously demonstrated, approximately 1 hour after exposure to GHRHa, followed by disappearance of pro-survival Bcl-2 at 2 hours. Preliminary time course analysis suggests that Bcl-2 remains reduced up to 4 hours after exposure.

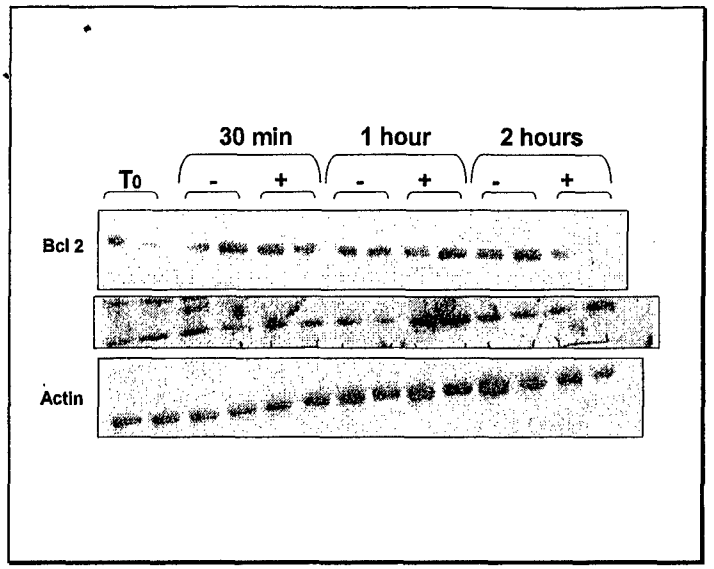

Figure 16: Antagonism of endogenous GHRH promotes activation of caspase 3, followed by disappearance of Bcl-2.

Caspase dependent cleavage of pro survival Bcl-2 has been reported. In myeloid leukemic cells, the addition of the caspase-1 inhibitor Z-VAD-FMK prior to treatment with etoposide prevented cleavage of the Bcl-2 protein[35]. Cleavage of Bcl-2 four hours after neocarzinostatin (NCS) treatment of PC-12 pheochromocytoma cells was prevented by the caspase-3 specific inhibitor, Ac-DEVD-CHO[36]. Similarly, removal of IL2, triggers caspase-dependent cleavage of Bcl-2[37-38]. While the experiments described here do not prove that the disappearance of the Bcl-2 protein is due to the activated caspases, the temporal relationship between the two events and the previously described caspase-dependant cleavage of Bcl-2 suggest that the disappearance of Bcl-2 following exposure of cells to GHRHa involves activation of the caspase cascade.

Taken together, the data presented here suggest that endogenous GHRH acts as a growth factor through activation of MAPK/ERK. At the same time, arguing backward from the effects of antagonism, the data suggest an anti-apoptotic action of GHRH through suppression of p38 activation of a caspase cascade (9>3>2) and consequent inhibition of Bcl-2 cleavage. Activation of the Jnk pathway may antagonize the effects of GHRH on the p38 pathway. Our tentative understanding of this pathway is illustrated in Figure 17.

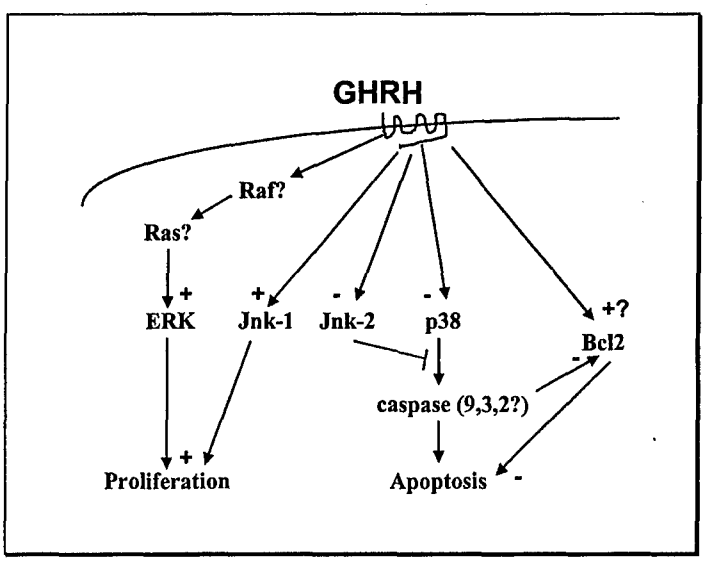

Figure 17: Proposed pathway for growth promoting and anti-apoptotic actions of endogenous GHRH in MDA231 cells.

We have now developed a culture paradigm in which culture medium is changed 2 hours prior to obtaining the baseline sample. Under these conditions, basal levels of ERK1/2 activation are

markedly suppressed, and rapid phosphorylation of ERK1/2 stimulation by GHRH can be demonstrated. Therefore, it has become possible to investigate the direct effects of exogenous GHRH and, thereby, understand more directly the effects of GHRH, rather than working backwards from the effects of inhibiting endogenous GHRH. As shown in Figure 18, the effect of GHRH on MDA231 cell numbers in DMEM without FCS was dose dependent, with a linear increase in cell numbers in response to logarithmically increasing doses of added GHRH..

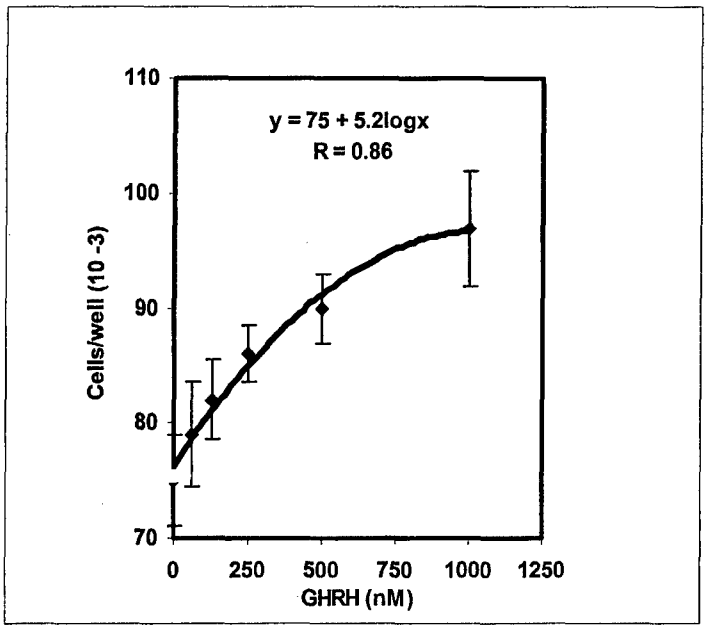

Figure 18. GHRH causes dose dependent cell proliferation of MDA 231 cells. Values represent the mean ± SEM; n = 8 replicates at each time point for each experiment, with results pooled from two independent experiments.

To examine more directly the effect of GHRH on cell proliferation, cells grown overnight in DMEM with no serum were treated with GHRH (1 μM), followed 2 hours later by addition of tritiated thymidine (Figure 19). When thymidine incorporation was measured 4 hours, 6 hours and 8 hours after the treatment of GHRH, there was a 40% increase in thymidine uptake in the GHRH treated cells relative to control cells at 6 hours and 49% increase at 8 hours, results similar to those previously reported in GH4 pituitary cells.

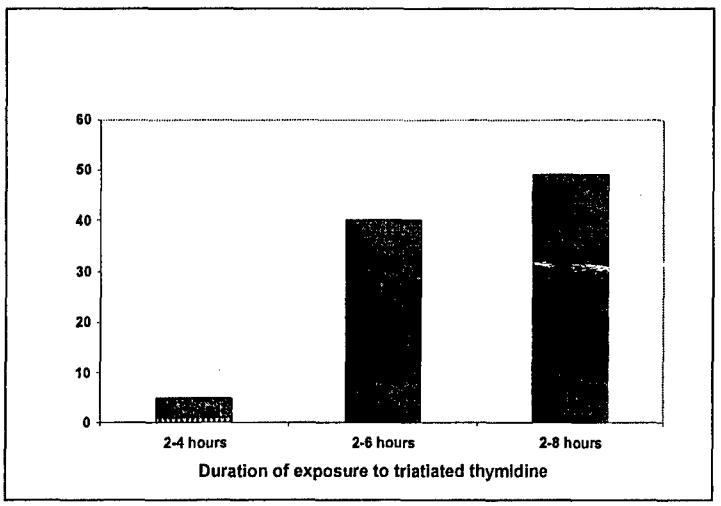

Figure 19. Incorporation of tritiated thymidine after treatment of MDA231 cells with GHRH. Results are percentage increase in thymidine incorporation relative to control, N = 8 well treatment.

To examine MAPK phosphorylation in response to GHRH, cells grown overnight were washed and incubated in serum-free DMEM for 1.5 hours, followed by addition of GHRH (0.1 nM to 1 μM) (Figure 20). As shown, GHRH stimulated robust and rapid phosphorylation of MAPK in MDA231 cells. The temporal pattern of phosphorylation was dependent on dose, as previously reported[27].

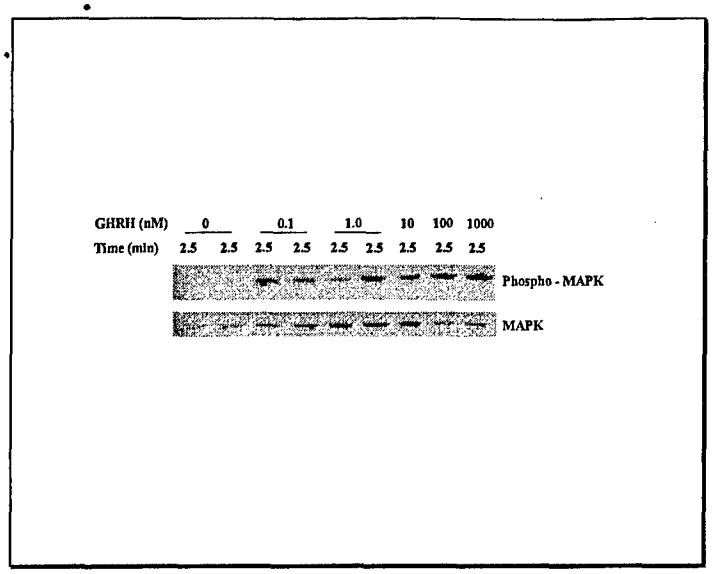

Figure 20. GHRH causes MAPK phosphorylation in MDA 231 cells.

To determine the signaling pathway involved in the stimulation of MAPK phosphorylation by GHRH, MDA 231 cells were transfected with pRSV, pZCR17N (dominant negative Ras) and pRSV-Raf-C4 (dominant negative Raf) and grown in DMEM with 10% FCS for 40 hours. At this time, the medium was replaced with serum free DMEM for 1.5 hours and the cells treated with GHRH as described above. As seen in Figure 21, inhibition of the ras and raf pathways effectively eliminated the phosphorylation of MAPK caused by GHRH, though there was a small increase in basal MAPK phosphorylation in the ras and raf inhibited cells.

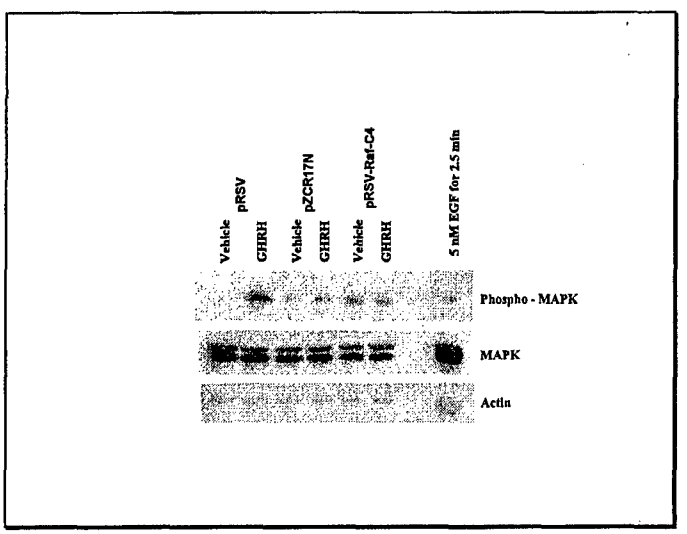

Figure 21 ***Raf and Ras proteins are required for the GHRH caused phosphorylation of MAPK in MDA 231 cells***

In addition to inhibition of GHRH stimulated MAPK phosphorylation, transfection with dominant negative Ras and dominant negative Raf constructs also prevented GHRH stimulation of MDA 231 cell proliferation, as shown in Figure 22.

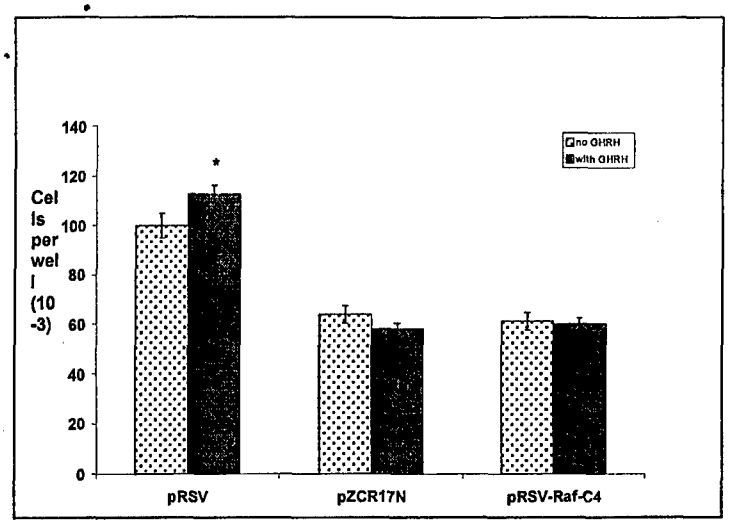

Figure 22. Inhibition of raf and ras pathways prevents GHRH stimulation of cell proliferation Values represent the mean ± SEM; n = 8 replicates.

To further investigate the timing of dependence of GHRH signaling on Raf and MAPK, we conducted two separate sets of experiments. In the first, the over night medium was replaced with DMEM with no serum. The inhibitors for the Raf1 kinase (5-Iodo-3[(3,5-dibromo-4hydroxyphenyl)methylene]-2-indolinone) and MAPK (PD 98059) (2-(2'-amino-3'-methoxyphenyl)-oxanaphthalen-4-one)) were added 20 minutes prior to the addition of 1μM GHRH and cells counted 24 hours later. As seen in Figure 23, inhibitors of Raf1 kinase and MAPK added prior to GHRH prevented GHRH-induced stimulation of cell proliferation.

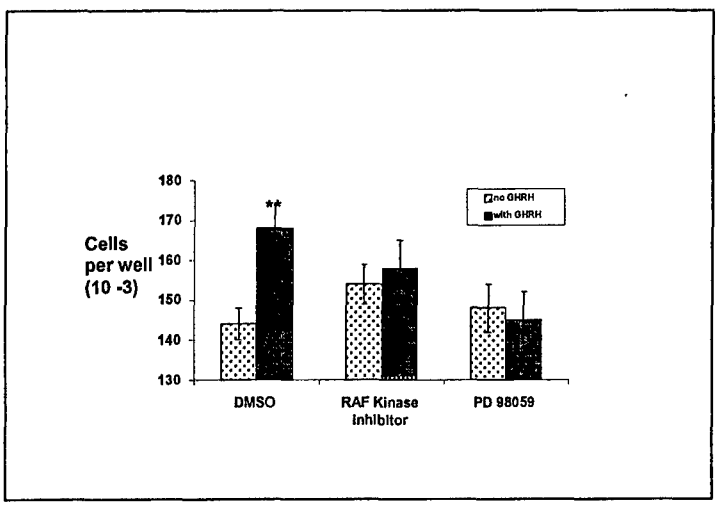

Figure 23. Inhibition of Raf and MAP Kinase before the addition of GHRH prevents the GHRH caused cell proliferation. Values represent the mean ± SEM; n = 8 replicates.

In a second set of experiments, the raf1 kinase and MAPK inhibitor were added immediately after the addition of GHRH (Figure 24). Comparison of the results of these two experiments indicates that when the inhibition of raf and MAPK after the addition of GHRH does not prevent GHRH-induced cell-proliferation. This suggests that there is rapid and transient activation of the intracellular signal requiring raf and MAPK following exposure to GHRH.

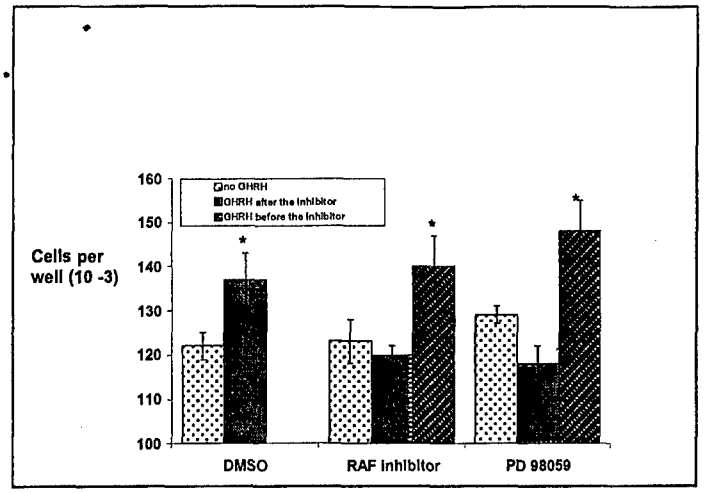

Figure 24. Inhibition of RAF 1 kinase or MAP kinase after the addition of GHRH does not prevent the mitotic activity of GHRH. Values represent the mean ± SEM; n = 8 replicates.

Previous studies have suggested the presence of an endogenous rhythm of GHRH release from breast cell lines. If this is the case and GHRH acts in an autocrine fashion to stimulate breast cell signal pathways, we hypothesized that there would be an endogenous cycle of both GHRH secretion from these cells, as well as an endogenous rhythm of MAPK phosphorylation in response to this autocrine stimulus. In order to test this hypothesis, MDA 231 cells were harvested at 20 minute intervals beginning 16 hours after. We then examined the phosphorylation of MAPK. As shown in Figure 25, there was a periodic fluctuation in phosphorylation of MAPK, with increases occurring approximately every 80 minutes.

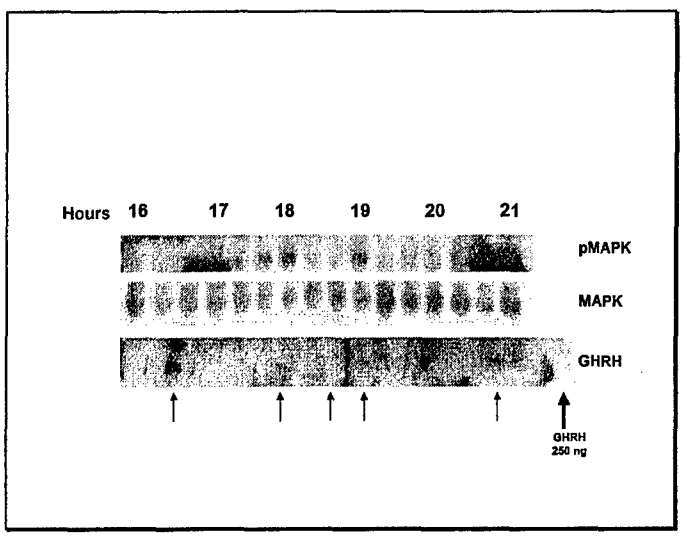

Figure 25. Cyclic phosphorylation of MAPK in MDA231 cells is correlated with production of endogenous GHRH. Arrows indicate times of detectable GHRH in cell lysate. Control lane contains authentic GHRH 250 ng for comparison.

Furthermore, GHRH was detected in the lysate just prior to the increased MAPK phosphorylation. Estimation by densitometry indicates that GHRH amounts at 16.7, 19, 20.7 and 21 hours were approximately 1000, 430, 500 and 388 ng respectively, or 100 -200 fM. Protein assays conducted on MDA 231 lysate plated in a similar fashion had an average protein concentration of 80 µg/20 µl after 16 to 21 hours (data not shown). Hence the amount of GHRH in the lysate ranges between 1250 – 2500 fM/mg of protein.

• To determine if these periodic increases in the pMAPK were causally related to the periodic release of GHRH, a similar set of MDA 231 cells were harvested every 20 minutes, but were treated with 10μM GHRH agonist PRL 2640, 20 minutes before harvesting. As shown in Figure 26, the application of the GHRH specific agonist resulted in a nearly complete reduction in the phosphorylation of MAPK in all instances, except for two sets of samplings soon after treatment with the antagonist. These results suggest, though do not prove, that the endogenous release of GHRH can, and does, provide a stimulus for MDA 231 cell proliferation.

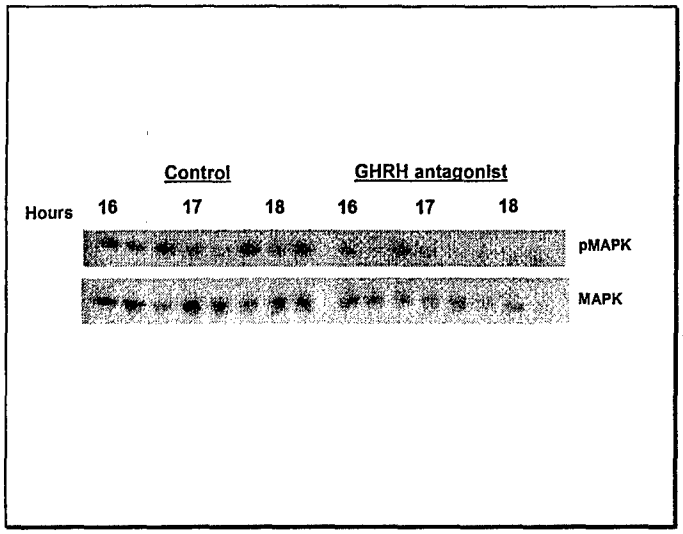

Figure 26. Cyclic phosphorylation of MAPK in MDA231 cells is inhibited by treatment with GHRH antagonist.

Somotostatin has previously been shown to inhibit the growth of human breast tumor cell lines[39]. Phosphorylation of MAPK by GHRH is inhibited in GH4 pituitary somatotroph cells by the presence of somatostatin[40]. We used the long-acting somatostatin analog, BIM 23014, to determine if somatostatin prevents GHRH-induced phosphorylation in MDA231 cells. As seen in Figure 27, pretreatment of MDA 231 cells with BIM 23014, prevented the phosphorylation of MAPK. The effect of somatostatin on GHRH-induced cell proliferation was determined by treating MDA 231 grown overnight in serum free medium with somatostatin before or after the addition of GHRH (Figure 12). As shown, pretreatment with somatostatin prevented the cell proliferation completely, while treatment with somatostatin following exposure to GHRH resulted in GHRH-induced cell-proliferation, though reduced compared to cells not exposed to somatostatin at all.

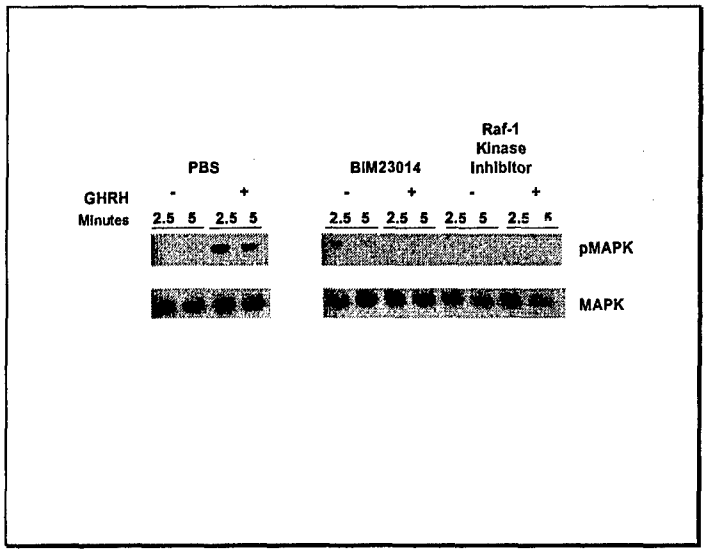

Figure 27. Somatostatin inhibits GHRH-induced MAPK phosphorylation.

Task 3: **To define the sequences of GHRH and GHRH receptor (GHRHr) promoters responsible for expression of these genes in breast cancer cells. (Months 24-36)**

Limited progress has been made on this task due to focus on Task 1 and 2.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstration that GHRH promotes MAPK phosphorylation/activation in MDA231 cells.
- Demonstration that GHRH promotes proliferation of MDA231 cells.
- Demonstration that GHRH-incuded activation of MAPK and stimulation of cell proliferation occurs through ras/raf dependent signaling.
- Demonstration that disruption of endogenous GHRH action in MDA231 breast cancer cells with the GHRH antagonist, GHRHa, results in:
  - transient, reversible, and dose-dependent decrease in cell proliferation
  - transient and reversible decrease in thymidine kinase uptake, indicating decreased cellular mitosis
  - increased cellular apoptosis
- Demonstration that disruption of endogenous GHRH action in MDA231 breast cancer cells with the GHRH antagonists, GHRHa or PRL2140, results in:
  - transient activation of P38 kinase
  - transient activation of caspase 3
  - loss of Bcl-2 expression following activation of caspase 3
  - activation of Jun kinase
- Demonstration that inhibition of GHRHa activation of P38 kinase results in:
  - prevention of GHRHa effect on MDA231 cell number
  - prevention of GHRHa-induced apoptosis in MDA231 cells
- Demonstration that inhibition of GHRHa activation of caspase 2,3 and 9 results in prevention of GHRHa induced cell loss.
- Demonstration that inhibition of GHRHa activation of Jun kinase 2 results in amplification of the effect of GHRHa on MDA231 cell number
- Demonstration of endogenous rhythm of GHRH production by MDA231 cells
- Demonstration of correlation of endogenous GHRH rhythm with cyclical MAPK phosphorylation in MDA231 cells.
- Demonstration of potential GHRH/somatostatin autocrine interaction to regulate MDA231 cell proliferation.

REPORTABLE OUTCOMES

Zeitler P, Siriwardana G. Antagonism of endogenous growth hormone-releasing hormone leads to reduced proliferation and increased apoptosis in MDA231 breast cancer cells. Endocrine 18:85-90, 2002

Zeitler P, Siriwardana G, Coy D, Bradford A. Growth Hormone Releasing Hormone (GHRH) Induces Proliferation of MDA 231 Breast Cancer Cells via the Ras, Raf , MAP Kinase Pathway. Submitted for review, J. Biol Chem.

Zeitler P, Siriwardana G. Antagonism of endogenous growth hormone releasing hormone promotes apoptosis in MDA231 breast cancer cells: Activation of p38 and the caspase pathway. In preparation.

Zeitler PS, Siriwardana G. Growth hormone-releasing hormone (GHRH) stimulates proliferation and decreases apoptosis in breast cancer cell lines. Presented at the 82nd Annual meeting of the Endocrine Society, Toronto, ON, 2000

Zeitler PS, Siriwardana G. Signaling by endogenous growth hormone-releasing hormone (GHRH) in MDA231 breast cancer cell lines. Presented at Era of Hope, Orlando FL 2002

CONCLUSIONS

This project has expanded our understanding of the pathway by which endogenous GHRH production promotes maintenance and proliferation of MDA231 cell populations. Furthermore, these studies begin to delineate the mechanism by which the antagonism of the endogenous GHRH autocrine/paracrine system in MDA231 breast cancer cells leads to inhibition of cell proliferation, as well as increased cellular apoptosis, the combination of which leads to decreased cell number. These studies clarify the intracellular signaling pathways through which the actions of GHRH on MDA231 cells are mediated and allows us to develop a preliminary and testable model for GHRH biology in breast neoplasia, presented in Figure 17.

The emerging picture of the pathway by which GHRH promotes growth and inhibits apoptosis in breast cancer cell lines furthers our understanding of the previously demonstrated actions of GHRH antagonists to inhibit breast cancer growth in vitro and in vivo. More importantly, this understanding begins to suggest ways in which GHRH antagonists might fit into therapeutic regimens, as pro-apoptotic agents in their own right or as adjuvant agents supporting the action of traditional anti-neoplastics. For example, overexpression of Bcl-2 may be related to drug resistance in many tumors and is an indicator of poor prognosis[41-46]. Since GHRH and/or its receptor have been reported in many cancers[17, 47-48], the ability of GHRH antagonists to promote Bcl-2 cleavage could provide a novel approach to drug resistance in cancers that express the GHRH receptor.

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Appendix

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Antagonism of Endogenous Growth Hormone–Releasing Hormone Leads to Reduced Proliferation and Apoptosis in MDA231 Breast Cancer Cells

Philip Zeitler and Gamini Siriwardana

Division of Endocrinology, Department of Pediatrics, University of Colorado Health Science Center and The Children's Hospital, Denver, CO

AU: shorten abstract to no more than 200 words

GHRH, in addition to stimulating the release of growth hormone (GH) from the pituitary, acts as a trophic factor for pituitary somatotrophs. Growth hormone–releasing hormone (GHRH) is also expressed outside of the hypothalamic/pituitary axis—in the gonads, gastrointestinal tract, pancreas, thymus, and lymphocytes, as well as in tumors of the pancreas, lung, central nervous s[illegible]m, and breast. However, the physiologic role of extrahypothalamic GHRH is unknown. Since GHRH has mitogenic effects in some extrapituitary sites, we examined the hypothesis that GHRH functions as an endogenous autocrine/paracrine growth factor in neoplastic breast tissue. MDA231 cells were grown under standard conditions and GHRH receptor expression was demonstrated by polymerase chain reaction amplification. The effect of disrupting endogenous GHRH on cell growth and apoptosis was examined through the use of a competitive GHRH antagonist, [*N*-acetyl-Tyr1, D-Arg2] fragment 1–29Amide (GHRHa) (1–3 μ*M*). Cell proliferation was determined by direct cell counting and tritiated thymidine incorporation. Apoptosis was analyzed by examination of DNA laddering and nuclear condensation. Exposure of MDA231 cells to GHRHa resulted in a dose-dependent, transient, and reversible decrease in cell number and proliferation rate. Furthermore, GHRHa resulted in a transient and reversible decrease in tritiated thymidine uptake, indicating decreased cellular proliferation rate. Conversely, exposure of MDA231 cells to GHRHa led to a marked and dose-dependent increase in both DNA laddering and nuclear condensation, implying the promotion of apoptosis. These results indicate that disruption of endogenous GHRH action in MDA231 cells results in both decreased cellular proliferation and increased apoptosis. Taken together, the findings suggest that endogenous GHRH acts as an autocrine/paracrine factor in the regulation of growth of at least some breast cancer cell types.

Key Words: Growth hormone–releasing hormone; neoplasia, breast; growth factor; apoptosis; autocrine/paracrine growth control.

Introduction

The hypothalamic neuropeptide growth hormone–releasing hormone (GHRH) stimulates growth hormone (GH) synthesis and secretion from the pituitary and is a critical trophic factor promoting development and proliferation of pituitary somatotrophs *(1–8)*. GHRH is also expressed in a limited set of other tissues, including lymphocytes, placenta *(9,10)*, gut *(11)*, kidney *(12)*, thymus *(13)*, and testis *(10,14)*, where it is assumed to play an autocrine/paracrine role. However, the physiology of extrahypothalamic GHRH has not been well studied, although mitogenic activity has been reported in lymphocytes and testicular germ cells *(15–17)*, suggesting that a trophic role for GHRH may not be unique to the pituitary somatotroph. Recently, we *(18)* and others *(19)* have demonstrated that GHRH activates the mitogen-activated protein kinase (MAPK) pathway, as well as cellular proliferation in somatotroph cell lines, providing a potential signaling framework for mitogenic actions.

GHRH is also known to be expressed in tumors of the central nervous system, lungs, and gastrointestinal (GI) tract *(20)*. Indeed, GHRH was originally isolated from pancreatic tumors, and ectopic secretion of GHRH is a well-described cause of acromegaly. More recently, expression has been demonstrated in tumors of the breast *(21)*, prostate *(22)*, ovary, and endometrium *(23–25)*. Furthermore, GHRH receptor antagonists (GHRHa) have been reported to have antitumorigenic activity in a variety of transformed human cell lines, including GI tract, renal, prostate, ovarian, and breast *(26–31)*. However, the mechanism of action of these antagonists to inhibit tumor growth, as well as the underlying role of GHRH itself in these tumors, remains unclear.

To study GHRH biology in extrahypothalamic tumors, we were interested in establishing an in vitro model amenable to molecular dissection. Since breast cancer cell lines

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Author to whom all correspondence and reprint requests should be addressed: Dr. Philip Zeitler, Division of Endocrinology, The Children's Hospital, 1056 East 19th Avenue, Denver, CO 80218. E-mail: Phil.Zeitler@uchsc.edu

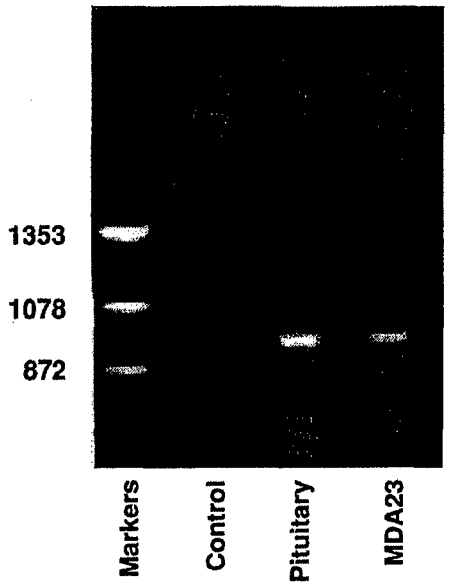

Fig. 1. RT-PCR amplification of total RNA from a human pituitary tumor and MDA231 cells. RT-PCR amplification (35 cycles) was performed using 1 μg of total RNA prepared from MDA231 cells and a human pituitary tumor and primers designed to amplify a 953-bp segment of the hGHRH receptor as described in Materials and Methods. Cloning and direct sequencing confirmed the identity of the transcript as hGHRH receptor mRNA.

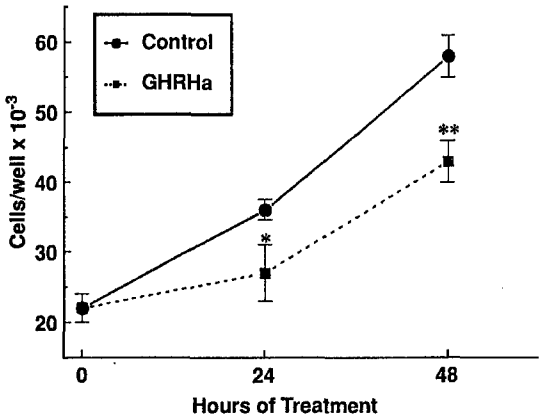

Fig. 2. Effect of GHRHa on MDA231 cell counts in vitro. MDA231 cells were plated at 8000/cm in 96-well plates and allowed to attach overnight in Dulbecco's modified Eagle's medium (DMEM)/2% fetal calf serum (FCS). The medium was replaced with DMEM without serum, and the competitive GHRH antagonist GHRHa was applied for 1 h as described in Materials and Methods. The medium was then brought to 2% FCS and allowed to incubate until the indicated times. In all experiments, control cells were treated with the same final concentration of vehicle alone. At the time of counting, the medium was aspirated, and the cells were removed following treatment with 50 μL of trypsin and resuspended in 150 μL of PBS. Four 0.1-μL samples of each well were counted by hemocytometer, with eight replicates per treatment. Values represent the mean ± SEM; $n = 8$ replicates at each time point for each treatment. $*p < 0.05$; $**p < 0.01$.

AU: OK?

express endogenous GHRH *(21,32)*, we first demonstrated that these cell lines also express the GHRH receptor and then examined the effect of disrupting the autocrine/paracrine actions of GHRH on proliferation and apoptosis of these cells using a GHRH receptor antagonist.

Results

As shown in Fig. 1, reverse transcriptase polymerase chain reaction (RT-PCR) of total RNA from MDA231 cells using hGHRHr-specific primers detected the presence of a 953-bp transcript identical in size to that present in human pituitary tissue. No transcript was present in amplifications lacking input RNA (control lane). Cloning and direct sequencing confirmed the identity of the transcript as full-length hGHRH receptor mRNA. Western blot analysis confirmed previous reports of immunoreactive GHRH in extracts from MDA231 cells *(32)*.

The effect on cell growth of disruption of endogenous GHRH signaling was examined using the hGHRH antagonist, [*N*-acetyl-Tyr1, D-Arg2] fragment 1-29Amide (Sigma, St. Louis, MO). As shown in a representative experiment (Fig. 2), a single treatment of MDA231 cells with 3 μ*M* antagonist resulted in an approx 25% decrease in cell number after 24 h. Subsequently, cell numbers increased in parallel with control cells, indicating that the effect is transient and reversible. A second treatment after 24 h led to an additional 24 h of inhibition of the increase in cell number. This inhibition by GHRHa was also dose dependent. As shown in a representative experiment (Fig. 3), exposure for 24 h to GHRHa resulted in decreases in cell number ranging from 6% at 40 n*M* to 25% at 5 μ*M*. Higher doses did not lead to decreases >25%.

To determine the mechanism responsible for the reduction in cell number caused by exposure to GHRHa, the effect of GHRHa on measures of cellular proliferation and mitosis was examined. As shown in a representative experiment (Fig. 4), exposure of MDA231 cells to a single dose of 3 μ*M* GHRHa resulted in a rapid and transient decline in thymidine uptake followed by uptake parallel to control cells. This change in thymidine uptake indicates a decrease in DNA synthesis and suggests a decrease in cellular proliferation. When cells were exposed to a second dose of GHRHa after 4 h, tritiated thymidine uptake was inhibited for an additional 4 h followed by recovery and uptake parallel to control cells.

To evaluate whether antagonism of endogenous GHRH also decreased cell counts through promotion of apoptosis, the effect of GHRHa on apoptosis was determined using two independent techniques. As shown in Fig. 5, exposure of MDA231 cells to 3 μ*M* GHRHa led to a marked increase in DNA laddering compared to vehicle alone. In addition,

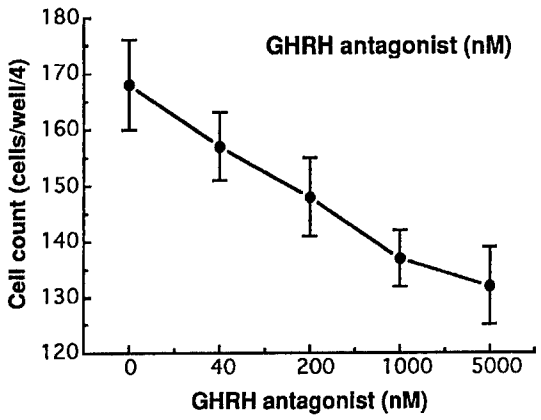

Fig. 3. Dose-dependent effect of GHRHa on MDA231 cell counts in vitro. MDA231 cells were plated at 8000/cm in 96-well plates and allowed to attach overnight in DMEM/2% FCS. The medium was replaced with DMEM without serum, and the competitive GHRH antagonist GHRHa was applied for 1 h as described in Materials and Methods. The medium was then brought to 2% FCS and allowed to incubate until the indicated times. In all experiments, control cells were treated with the same final concentration of vehicle alone. At the time of counting, the medium was aspirated, and the cells were removed following treatment with 50 μL of trypsin and resuspended in 150 μL of PBS. Four 0.1-μL samples of each well were counted by hemocytometer, with eight replicates per treatment. Values represent the mean ± SEM; n = 8 replicates at each time point for each treatment.

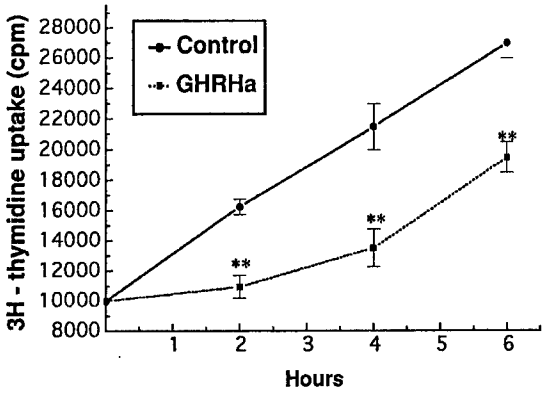

Fig. 4. Effect of GHRHa on ^{3}H-thymidine uptake by MDA231 cells in vitro. MDA231 cells were plated at 8000/cm in 96-well plates and allowed to attach overnight in DMEM/2% FCS. The medium was replaced with DMEM without serum and the competitive GHRH antagonist GHRHa was applied for 1 h as described in Materials and Methods. The medium was then brought to 2% FCS. Four hours after GHRHa treatment, 0.8 μL of ^{3}H-thymidine was added to each well. At the indicated times, cells were washed thrice with 150 μL of phosphate-buffered saline (PBS), followed by 25 μL of 10% trichloroacetic acid (TCA). After 5 min, 100 μL of 0.1 *M* NaOH was added followed by 27.5 μL of 0.*1M* HCl. The entire content of the well was transferred to scintillation counter tubes and counted for 10 min. Values represent the mean ± SEM; n = 8 replicates at each time point for each treatment. $^{**}p < 0.01$.

exposure of MDA231 cells to 3 μ*M* GHRHa for 24 h increased the frequency of appearance of condensed nuclei after staining with Hoechst dye. To quantify this increase, the number of condensed nuclei present in a ×100 field (four fields per slide, eight slides per treatment) was counted. As shown in Fig. 6, after 24 h in the presence of 3 μ*M* GHRHa, the frequency of apoptotic cells increased by 60–75% in independent experiments. However, even after the increase in apoptotic frequency following GHRHa treatment, the overall rate of apoptosis remained limited, with approx 5% of cells in a field of 2000 demonstrating signs of apoptosis at 24 h, a rate consistent with the decrease in cell numbers at 24 h.

Discussion

The data presented here indicate that antagonism of the endogenous GHRH autocrine/paracrine system in MDA231 breast cancer cells leads to inhibition of cell proliferation, as well as increased cellular apoptosis, the combination of which leads to decreased cell number. The effect of GHRH antagonist is dose-dependent, transient, and reversible. These results are consistent with previous demonstrations of an inhibitory effect of GHRH antagonists on a variety of reproductive and GI tract tumors, extending these observations to provide initial information regarding the mechanism of the effect of GHRH antagonists.

The decrease in cell numbers seen in the MDA231 cells exposed to GHRH antagonist is unlikely to be owing to nonspecific toxicity. The effects of the antagonist are transient and completely reversible, suggesting a physiologic rather than catastrophic event. Furthermore, the effect of the antagonist on cellular proliferation is relatively modest even at maximal doses, suggesting that the effect is limited to certain cells, perhaps in a particular physiologic state or position in the cell cycle, rather than a generalized toxic effect on all cells. Finally, the decrease in cell number is associated with DNA laddering and nuclear condensation, features characteristic of apoptosis rather than nonspecific cell death.

The implications of changes in thymidine uptake are arguable. On the one hand, a decrease in uptake may reflect decreased rates of DNA synthesis (i.e., reduced mitosis). Alternatively, decreased uptake may indicate reduced rates of DNA repair processes. However, in the current experiments, the association of decreased thymidine uptake with the subsequent reduction in cell number strongly suggests that exposure to GHRH antagonist is promoting a decrease in the rate of mitosis and cell proliferation. Furthermore, while it is conceivable that the decrease in thymidine uptake reflects the loss of DNA synthesis by cells undergoing apoptosis, the degree of reduced cell number appears to exceed what can be accounted for by apoptosis alone, implying that

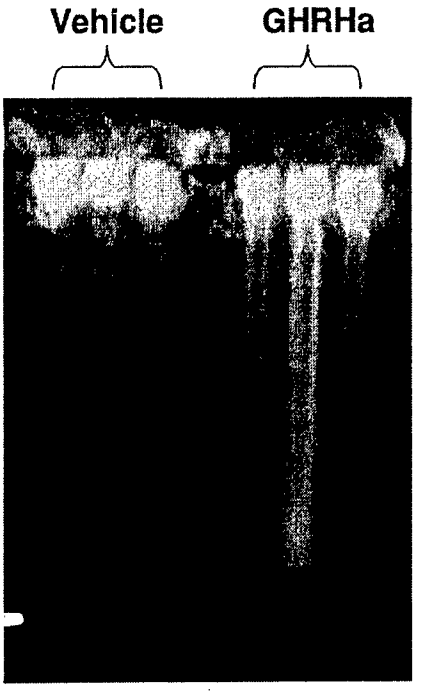

Fig. 5. Effect of GHRHa on DNA laddering in MDA231 cells. MDA231 cells were grown as described in 3.5-cm plates overnight. Following treatment with GHRHa as described, the volume of medium was increased to 3 mL. Cells were harvested 12 h after GHRHa treatment, pelleted by centrifugation, and washed once in PBS. Cells were lysed in 20 µL of lysis buffer (50 m*M* Tris, pH 8.0; 10 m*M* EDTA; 0.5% sodium dodecyl sulfate [SDS]; 0.5 mg/mL of proteinase K) and heated to 50°C for 1 h. The mixture was then heated to 90°C for 3 min to deactivate the proteinase K, treated with 10 µL of RNase A to a final concentration of 0.5 µg/mL in TE, and heated to 50°C for 1 h. Samples were separated by electrophoresis through 2% agarose with ethidium bromide and visualized (Alphaimager). Each lane represents the total DNA sample obtained from a single replicate, with three replicates per treatment.

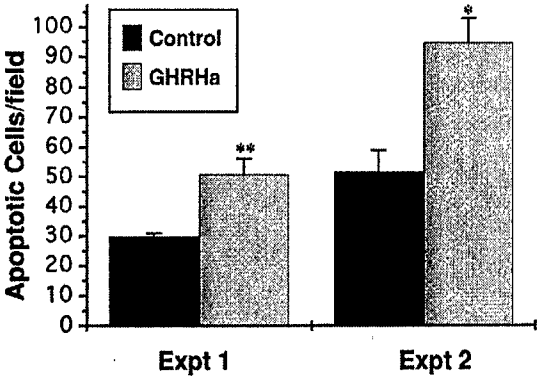

Fig. 6. Effect of GHRHa on nuclear condensation of MDA231 cells in vitro. Cells were grown as described in 100 µL of DMEM/2% FCS on chamber slides overnight. GHRHa was added in 80 µL of DMEM and allowed to incubate for 1 h. The medium was then brought to 2% FCS. Twenty-four hours after GHRHa treatment, cells were washed briefly with PBS, fixed in 4% paraformaldehyde for 10 min, followed by 70% EtOH in glycine buffer for 10 min at –20°C. Cells were then washed in PBS, incubated with Hoechst dye (8 µg/mL) for 15 min at room temperature, and rinsed three times in PBS. Slides were then masked and cells visualized by fluorescent microscopy and apoptotic cells counted (four fields per slide, eight slides per treatment). Values represent the mean ± SEM in two independent experiments. $*p < 0.05$; $**p < 0.01$.

at least a portion of the cell number reduction is a consequence of reduced cellular proliferation.

The concept of GHRH as a promoter of cellular proliferation is not in itself novel. Within the hypothalamic pituitary axis, extensive evidence supports the role of GHRH in the development and proliferation of GH-secreting somatotrophs. GHRH stimulates the expression of both the GH gene and c-*fos*, and enhances somatotroph proliferation in vitro *(4–6,18,33–35)*. Long-term exposure to GHRH in vivo results in somatotroph hyperplasia in animals *(36,37)* and humans *(38,39)*. Conversely, rats in which GHRH action is transiently impaired during the neonatal period have reduced pituitary size and somatotroph cell number *(7,40–42)*. Similarly, resistance to GHRH action, as in the *lit* mouse *(43, 44)* or *dw* rat *(2)*, or congenital absence of GHRH, as in the GSH-1 knockout mouse *(1)*, is associated with marked somatotroph hypoplasia.

However, the mechanism by which GHRH promotes cellular proliferation is unclear. In pituitary somatotrophs, analogs of cyclic adenosine monophosphate (cAMP) and somatotroph-targeted expression of cholera toxin in transgenic animals induce cellular proliferation in culture *(35,45)*. Conversely, GH-promoter driven overexpression of dominant negative CREB leads to somatotroph hypoplasia *(8)*, a finding interpreted to indicate that inhibition of the transcriptional effects of cAMP prevents the genomic and proliferative effects of GHRH. Recently, we *(18)* and others *(19)* have demonstrated that GHRH activates the MAPK pathway, as well as cellular proliferation in somatotroph cell lines. Furthermore, proliferation in response to GHRH was prevented by agents that prevent activation of MAPK, strongly implying that GHRH promotes proliferation, at least in part, through activation of the MAPK pathway.

The question of which receptor is transducing extrahypothalamic actions of GHRH has been somewhat controversial. In some cases, attempts at identifying the GHRH receptor in GHRH antagonist–responsive tumors has been unsuccessful *(21,29,46)*, and it has been suggested that GHRH may be acting through related vasoactive intestinal peptide or PACAP receptors *(47,48)*. However, in the case of MDA231 breast cancer cells, the presence of GHRH receptor mRNA suggests that the actions of GHRH on cellular proliferation are likely mediated by the GHRH receptor itself.

AU: define PACAP

In summary, the results of these experiments indicate that exposure of MDA231 breast cancer cells to a GHRH receptor antagonist in vitro results in reduced cell numbers. Furthermore, the experiments provide evidence that the decrease in cell number reflects both decreased cellular proliferation, as indicated by decreased tritiated thymidine uptake, and increased cellular apoptosis, as indicated by increased DNA laddering and nuclear condensation. Taken together, the data suggest that disruption of endogenous GHRH receptor signaling results in disruption of normal MDA231 cellular dynamics, leading to decreased proliferation and survival of the breast cancer cells. By extension, these results imply that endogenous GHRH supports MDA231 cell proliferation and inhibits apoptotic pathways.

Materials and Methods

Cell Culture

MDA231 cells, originally obtained from American Type Culture Collection, were grown to confluency under standard conditions in DMEM supplemented with 10% FCS. Prior to experiments, the cells were removed following treatment with PBS/2% EDTA; plated at 8000/cm in 96-, 24-, or 6-well plates; and allowed to attach overnight in DMEM/2% FCS. For treatments, the medium was replaced with DMEM without serum and treatments applied for 1 h, following which medium was brought to 2% FCS and maintained until harvest. The competitive GHRH antagonist GHRHa was dissolved in 2% acetic acid/1% insulin-free bovine serum albumin to a stock concentration of 1 m*M*. In all experiments, control cells were treated with the same final concentration of vehicle alone.

RT-PCR Amplification

Total RNA was prepared from MDA231 cells and a human pituitary tumor using commercial reagents. RT-PCR amplification (35 cycles) was performed using 1 μg of total RNA from each tissue and primers designed to amplify a 953-bp segment of the hGHRH receptor as previously described *(49)*.

Cell Counts

Cells were grown as described in 100 μL of DMEM/2% FCS in a 96-well plate overnight. GHRHa was added in 80 μL of DMEM and allowed to incubate for 1 h. The medium was then brought to 2% FCS and allowed to incubate until the indicated times. At the time of counting, the medium was aspirated, and the cells were removed following treatment with 50 μL of trypsin and resuspended in 150 μL of PBS. Four 0.1-μL samples of each well were counted by hemocytometer, with eight replicates per treatment.

Tritiated Thymidine Uptake

Cells were grown as described in 100 μL of DMEM/2% FCS in a 96-well plate overnight. GHRHa was added in 80 μL of DMEM and allowed to incubate for 1 h. The medium was then brought to 2% FCS. Four hours after GHRHa treatment, 0.8 μL of ^{3}H-thymidine was added to each well. At the indicated times, cells were washed thrice with 150 μL of PBS, followed by 25 μL of 10% TCA. After 5 min, 100 μL of 0.1 *M* NaOH was added followed by 27.5 μL of 0.*1M* HCl. The entire content of the well was transferred to scintillation counter tubes and counted for 10 min. Each treatment was examined in eight replicates.

DNA Ladder

Cells were grown as described in 3.5-cm plates overnight. Following treatment with GHRHa as described, the volume of medium was increased to 3 mL. Cells were harvested 12 h after GHRHa treatment, pelleted by centrifugation, and washed once in PBS. Cells were lysed in 20 μL of lysis buffer (50 m*M* Tris, pH 8.0; 10 m*M* EDTA; 0.5% SDS: 0.5 mg/mL of proteinase K) and heated to 50°C for 1 h. The mixture was then heated to 90°C for 3 min to deactivate the proteinase K, treated with 10 μL of RNase A to a final concentration of 0.5 μg/mL in TE, and heated to 50°C for 1 h. Samples were separated by electrophoresis through 2% agarose with ethidium bromide and visualized (Alphaimager).

Hoechst Staining

Cells were grown as described in 100 μL of DMEM/2% FCS on chamber slides overnight. GHRHa was added in 80 μL of DMEM and allowed to incubate for 1 h. The medium was then brought to 2% FCS. Twenty-four hours after GHRHa treatment, cells were washed briefly with PBS, fixed in 4% paraformaldehyde for 10 min, followed by 70% EtOH in glycine buffer for 10 min at –20°C. Cells were then washed in PBS, incubated with Hoechst dye (8 μg/mL) for 15 min at room temperature, and rinsed three times in PBS. Slides were masked so that the reader would be unaware of the treatment exposure, cells were visualized by fluorescent microscopy, and apoptotic cells were counted (four fields per slide, eight slides per treatment).

Statistical Analysis

Where indicated, data were analyzed by one-way analysis of variance followed by post-hoc analysis with the Neuman-Keuls test.

Acknowledgments

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SIGNALING BY ENDOGENOUS GROWTH HORMONE RELEASING HORMONE (GHRH) IN MDA 231 BREAST CANCER CELL LINES

Philip S. Zeitler MD, PhD and Gamini Siriwardana PhD

Department of Pediatrics, University of Colorado Health Sciences Center, Denver, CO 80226

Phil.Zeitler@uchsc.edu

GHRH, a pituitary growth factor, is expressed outside of the hypothalamic/pituitary axis and promotes mitosis in some systems. GHRH and its receptor are expressed in breast cancer cell lines and antagonism of endogenous GHRH inhibits proliferation and promotes apoptosis of MDA231 cells in vitro. To clarify the intracellular signal pathways that mediate the actions of GHRH on breast cancer cells, we examined the effect on intracellular signaling of disruption of endogenous GHRH. **Methods:** MDA 231 cells were grown in (DMEM) with 10% FCS overnight. The medium was then changed to fresh DMEM without FCS and cultures exposed to GHRH (1 μM) or antagonist ([N-Acetyl-Tyr, D-Arg] - Fragment 1 - 29 Amide (GHRHa) 5 μM). In some experiments, cultures were pretreated with pharmacologic inhibitors of intracellular signaling pathways. **Results:** Treatment of MDA231 cells with GHRH promoted a 12-25% increase in cell numbers after 24 hours. This increase was prevented by preincubation of cells with the MAP kinase inhibitor, PD98095. Antagonism of GHRH led to a 12 - 20 % reduction of MDA 231 cell numbers 24 hours after treatment, accompanied by a reduction in thymidine incorporation and appearance of a characteristic apoptotic ladder. Phosphorylation of p38 MAP kinase was observed 15 minutes after treatment of MDA231 cells with GHRHa. Inhibition of p38 MAP kinase with 10 μM SB20586 prevented both the reduction of cell number and the antagonist-induced apoptotic ladder. Exposure to GHRHa was also associated with reduction in the phosphorylation of Jun Kinase 2 (Jnk2) but not Jnk-1, compared to baseline. Transient transfection of dominant negative Jnk –2, but not JNK –1, enhanced GHRHa-induced cell reduction - up to 50%. In addition, western analysis after antagonist treatment demonstrated the appearance of active forms of caspase 2 and 3. No change was seen in activation of other caspases. Blocking caspase 2 activity with the inhibitor Z-VDVAD-FMK prevented antagonist-induced cell reduction. Finally, Bcl-2 protein levels were reduced in antagonist-treated cells. **Conclusions:** These results suggest that endogenous GHRH promotes proliferation of MDA 231 cells via activation of MAP kinase and inhibits apoptosis through activation of Jnk2 and dephosphorylation of p38 kinase.

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